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Research Paper

Absorption enhancement studies of clopidogrel hydrogen sulphate in rat everted gut sacs

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Keywords
clopidogrel; everted gut sac; P-glycoprotein; solid dispersion; solubility

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Abstract

Objectives Clopidogrel, a thienopyridine antiplatelet agent, is a poor aqueous soluble compound and a P-glycoprotein (P-gp) efflux pump substrate. These two factors are responsible for its incomplete intestinal absorption. In this study, we have attempted to enhance the absorption of clopidogrel by improving its solubility and by inhibiting intestinal P-gp activity.

Methods Solubility enhancement was achieved by preparing solid dispersions. Quinidine and naringin were selected as P-gp inhibitors, whilst tartaric acid was selected as the intestinal absorption enhancer. Absorption studies were performed using the everted gut sac model prepared from rat jejunum. The determination of clopidogrel was performed by high performance liquid chromatography.

Key findings We noticed an enhancement of clopidogrel absorption by improving its solubility or by inhibiting the P-gp activity. The greatest results were obtained for solid dispersions in the presence of P-gp inhibitors at their highest concentrations, with an absorption improvement of 3.41- and 3.91-fold for naringin (15 mg/kg) and quinidine (200 μM), respectively. However, no clopidogrel absorption enhancement occurred in the presence of tartaric acid.

Conclusions Naringin, a natural compound which has no undesirable side effects as compared with quinidine, could be used as a pharmaceutical excipient in the presence of clopidogrel solid dispersions to increase clopidogrel intestinal absorption and therefore its oral bioavailability.

Introduction

Intestinal absorption is considered a key factor for the bioavailability of oral dosage forms. Several factors can affect the transfer process of drug compounds across the intestinal epithelial mucosa, such as physicochemical properties of the compound (hydrosolubility, lipophilicity, chemical nature, pK_a, ionic charge, molecular weight, etc.), the formulation aspect of the oral dosage form (e.g. particle size, polymorphism, tablets, capsules, solutions) and the physiological properties of the gastrointestinal (GI) tract (gastric emptying, surface area available for the absorption process, level of vascularization, blood flow, GI motility, pH values in the different regions of the GI tract, intestinal enzymatic systems etc.).[1] It is well known that influx and efflux transporters expressed on the brush-border membrane of the epithelial intestinal cells have a considerable impact on drug absorption. Influx transporters are known to facilitate drug absorption, whereas efflux transporters, such as P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRP1 and MRP2) and breast cancer resistance protein (BCRP) are known to restrict the absorption of a wide variety of drug compounds administered orally.[2]

Clopidogrel is a thienopyridine antiplatelet and antithrombotic agent that inhibits ADP-mediated platelet activation and aggregation by selectively and irreversibly blocking platelet purinergic P2Y12 receptors. It is an inactive prodrug that needs biotransformation in the liver catalysed by cytochrome P450 (CYP) 3A4 iso-enzyme mainly and other hepatic CYP proteins to form the active thiol metabolite 2-oxo-clopidogrel. However, this metabolite represents a small proportion, since the majority of clopidogrel (approximately 85%) is hydrolysed by esterase into an inactive carboxylic acid derivative.[3,4] Previously, it was reported that there was a considerable inter-individual variability in the response to clopidogrel treatment, which may have been

attributed to several factors, viz. variability in clopidogrel absorption and hepatic metabolism, polymorphism of clopidogrel's P2Y12 receptor, etc.[4–8]

Oral administration of clopidogrel remains problematic since this drug compound has a low aqueous solubility and is a P-gp efflux transporter substrate.[6–9] These two key factors (poor water solubility and P-gp efflux pump) are well known to be responsible for the incomplete absorption of numerous orally administered drugs and, therefore, of the limited oral bioavailability of such molecules.[2,10,11]

Solubility enhancement remains a challenging problem in the pharmaceutical industry. Over the years, numerous strategies have been developed in an attempt to improve solubility of poorly water soluble compounds such as particle size reduction, complexation using complex forming excipients (e.g. cyclodextrin), nanotechnology approaches, and drug dispersion into hydrophilic polymer (e.g. solid dispersion approach).[12]

P-gp is a transmembrane protein which belongs to the superfamily of ATP-binding cassette transporters (ABCB1). It is the product of the multidrug resistance (*MDR*1) gene located on chromosomal locus 7q21. It has two ATP-binding domains and two sets of transmembrane domains. It is an energy-dependent efflux pump, normally expressed at the apical side of most cell types and is present in all barrier tissues including liver, blood–brain barrier, intestine, kidney, eyes and lungs.[13,14] Its expression level, at the apical surface of epithelial cells in the GI tract, increase progressively from the proximal (stomach, duodenum and jejunum) to the distal region (ileum and colon).[15] P-gp alters intestinal permeation of a wide range of hydrophobic and cytotoxic compounds by limiting the influx into cells and by facilitating drug efflux from intestinal cells back into the lumen.[13,14] Previously, it was reported that inhibiting P-gp may improve drug absorption across intestinal barriers.[13,14,16] Several substances are used as P-gp inhibitors. These molecules may act as high avidity substrates (e.g. verapamil, diltiazem) or block P-gp function by binding to it (e.g. sulphydryl-substitued purine).[17]

In this study we have attempted to enhance clopidogrel intestinal absorption by improving its solubility using solid dispersions with macrogol 6000 (polyethylene glycol 6000) and by inhibiting the intestinal efflux transporter P-gp using quinidine and naringin as P-gp inhibitors. We investigated the effect of tartaric acid, an absorption enhancer, on clopidogrel absorption. Permeability studies were undertaken using the in-vitro everted rat gut sac model.

Materials and Methods

Drug and chemical reagents

Clopidogrel hydrogen sulphate, pharmaceutical grade, was kindly provided by Medis Laboratories (Nabeul, Tunisia). Naringin, tartaric acid, macrogol 6000, glucose, $CaCl_2$, $MgCl_2$ and HCO_3Na, reagent grade, were purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany); Na_2HPO_4 and KH_2PO_4 were obtained from Sigma Aldrich Laborchemikalien GmbH (Seelze, Germany). NaOH, citric acid, KCl and NaCl were obtained from Chemi-Pharma (Le Bardo, Tunisia). Other chemicals used were of reagent grade. Organic solvents used for HPLC analysis were of analytical grade.

Buffer solutions

Buffer solutions were prepared according to the European Pharmacopoeia (5th edn).[18] To prepare the phosphate buffer solution pH 6.8, 77.3 ml Na_2HPO_4 (71.5 g/l) was mixed with 22.7 ml citric acid solution (21 g/l). The pH of the medium was adjusted using a citric acid solution (21 g/l) or NaOH (0.1 M). To prepare phosphate buffer solution pH 7.4, 250 ml KH_2PO_4 (0.2 M) was mixed with 393.4 ml NaOH (0.1 M). The pH of the buffer solution was adjusted to 7.4 using either HCl (0.1 M) or NaOH (0.1 M).

Preparation of the physical mixture

A physical mixture of clopidogrel and macrogol 6000 at five drug:polymer weight ratios (1 : 1, 1 : 3, 1 : 5, 1 : 7 and 1 : 9, w/w) were obtained by thoroughly mixing in a mortar the appropriate amounts of clopidogrel with macrogol 6000. The resulting mixture was sieved through a 250-μm sieve and stored at room temperature in a hermetically sealed glass bottle until use. The particle size fraction selected for this study was 125–250 μm.

Preparation of solid dispersions

Solid dispersions containing clopidogrel and macrogol 6000 in five drug:polymer weight ratios (1 : 1, 1 : 3, 1 : 5, 1 : 7 and 1 : 9, w/w) were prepared as follows:[19] The appropriate amounts of clopidogrel and macrogol 6000 were dissolved in ethanol (50 ml) and in water (5 ml), respectively. The solutions were mixed under magnetic stirring for 30 min and then the solvents were evaporated under reduced pressure in a rotavapor at 60°C. The formed solid dispersions were stored in a vacuum desiccator over silica gel for 48 h to eliminate the residual solvent. Before being used for the experiments, the samples were pulverized using a mortar and pestle, sieved through a 250-μm sieve and stored at room temperature in a hermetically sealed glass bottle until use. The particle size fraction of 125–250 μm was selected for the study.

Preparation of the everted gut sacs

The experiments were carried out on male Wistar rats (200–250 g; 8–10-weeks-old) purchased from Central Pharmacy of Tunisia (Tunis, Tunisia). The animals were kept hygienically

in an animal house under controlled conditions. They were housed in large polypropylene cages at air conditioned temperature (22–24°C) with a 12-h light/dark cycle with free access to water and food. Before starting in-vitro experiments rats were starved for 24 h but they had free access to water.

All the animal experiments were performed in accordance with the guidelines established by the European Union on Animal Care (CCE Council 86/609) and approved by the Ethics Committee of the University Hospital of Monastir.

The rats were anaesthetized with ether before the experiments and upon verification of the loss of pain reflex, a 3-cm midline longitudinal incision of the abdomen was made. The small intestine was rapidly removed and flushed three times with 9% NaCl solution at room temperature and immediately placed in 37 ± 0.5°C and oxygenated (O_2/CO_2, 95% : 5%) Tyrode buffer solution (containing in mM: 15 glucose, 11.90 HCO_3Na, 136.9 NaCl, 4.2 NaH_2PO_4, 2.7 KCl, 1.2 $CaCl_2$ and 0.5 $MgCl_2$). After harvesting this segment the animal was immediately killed by cervical dislocation.

The everted gut sacs were prepared from rat jejunum.[20] Briefly, this segment was quickly excised, stripped of adhering tissue, washed three times with Tyrode solution and cut into small sacs (5–6 cm in length). Each sac was gently everted over a glass rod of 2.5 mm in diameter, tied with a silk braided suture at one end, connected to a small cannula at the other end and a second tie was made using a braided silk suture. The everted gut sacs were filled with 600 µl Tyrode buffer solution (pH 7.4) at 37°C from the cannula using a 1-ml plastic syringe. After that, each sac was hung in a 20-ml test tube containing 15 ml degassed oxygenated medium at pH 6.8. Finally, the test tubes were incubated at 37 ± 0.5°C in an oscillating water bath (OLS 200, Grant instruments, Cambridge, UK) at 60 cycles/min and were continuously oxygenated by mixed gas ((95% O_2–5% CO_2).

To examine the effect of quinidine, naringin or tartaric acid on clopidogrel (232 µM) or clopidogrel-macrogol 6000 solid dispersions absorption, the mucosal medium was filled with Tyrode solution containing quinidine (10, 50 or 200 µM), naringin (5, 10, 15 mg/kg) or tartaric acid (5, 10 or 20 mM). At the appropriate time points, samples were withdrawn from outside and inside the sacs and kept for HPLC analysis. The experiments were repeated six times ($n = 6$) and drug absorption was expressed as a percentage.

Solubility study

Solubility studies were performed as follow:[21] Excess amounts of pure clopidogrel or clopidogrel-macrogol 6000 binary systems (physical mixtures, solid dispersions) at different drug:polymer weight ratios (1 : 1, 1 : 3, 1 : 5, 1 : 7 and 1 : 9 w/w) corresponding to 100 mg pure drug compound were accurately weighed and placed into flasks containing 10 ml pH 6.8 buffer solution. The samples were shaken at room temperature for 24 h, withdrawn and centrifuged at 6000 rev/min for 10 min. Samples (1 ml) from the supernatants were filtered with a syringe using a 0.45-µm filter, appropriately diluted with the buffer solution and analysed by HPLC at 230 nm. The experiment was repeated six times ($n = 6$). Results were expressed in µg/ml.

Calculation of the apparent permeability coefficients

Apparent permeability coefficient (P_{app}) was determined according to equation 1:[20]

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{AC_0} \quad (1)$$

Where P_{app} (cm/s) is the apparent permeability coefficient, dQ/dt (µg/s) is the amount of drug transported across the membrane per unit of time, A (cm^2) is the surface area available for permeation and C_0 (µg/ml) represents the initial concentration of the drug outside the everted gut sacs.

Calculation of the percentage of drug recovery and drug retained

The percentage of drug recovery (R%) was calculated according to equation 2:

$$R\% = \frac{C_{r,end} \times V_r + C_{d,end} \times V_d}{C_0 \times V_r} \times 100 \quad (2)$$

where $C_{r,end}$ and $C_{d,end}$ (µg/ml) are the drug concentrations measured at the end of the experiment inside and outside the sacs, respectively; $C_{d,0}$ (µg/ml) is the initial concentration of the drug outside the everted gut sacs; V_r and V_d (ml) are the volumes of the mucosal and the serosal media, respectively.

The percentage of drug retained (Ad%) on the intestinal tissues was determined according to equation 3:

$$Ad\% = 100 - R\% \quad (3)$$

Water flux measurement

The water flux, resulting from both water absorption and efflux in the jejunum segment was determined according to equation 4:[4,22]

$$\text{Water flux} = \frac{P_3 - P_2}{P_1} \quad (4)$$

Where P_1 is the weight of the sac empty and blotted, P_2 is the weight of the sac + 600 µl Tyrode solution (pH 7.4) and P_3 is the weight of the sac at the end of the experiment. The water flux was determined after 2 h of experiments and the results

were expressed in g water/g fresh intestine. Assays for the evaluation of water flux were repeated six times ($n = 6$).

Diffusion profiles studies

To compare diffusion profiles obtained during the experiments, we used a model-independent approach consisting on the calculation of a difference factor (f_1) and a similarity factor (f_2).[23]

f_1 calculates the percent difference between two curves at each time point and is a measurement of the relative error between the two curves. It was determined according to equation 5:

$$f_1 = \left\{\left[\sum_{t=1}^{n}|R_t - T_t|\right] \Big/ \left[\sum_{t=1}^{n} R_t\right]\right\} \cdot 100 \quad (5)$$

Where n is the number of time points; R_t is the diffusion value of the reference batch at time t; and T_t is the diffusion value of the test batch at time t.

f_2 is a logarithmic reciprocal square root transformation of the sum of squared error. It is a measurement of the similarity in percent diffusion between the two curves and was calculated as per equation 6:

$$f_2 = 50 \cdot \log\left\{\left[1 + (1/n)\sum_{t=1}^{n}(R_t - T_t)^2\right]^{-0.5} \cdot 100\right\} \quad (6)$$

Two curves were considered similar when the f_1 value was less than 15% and the f_2 value was greater than 50% curves.

Analytical method

The amounts of clopidogrel inside and outside the everted gut sac were assayed using a HPLC system (1100 series Hewlett Packard) equipped with a HP 1100 series photodiode array UV detector, a HP 1100 series vacuum degasser, a HP 1100 series quaternary pump and a HP 1100 series manual injector using a rheodyne model 7725i 7-port sample injection valve. The HP 1100 series modules were controlled through HP ChemStation software. Samples from inside or outside the everted gut sac media were withdrawn, filtered through a 0.45-μm membrane filter and 20-μl samples were injected into the HPLC system. HPLC analysis of samples was performed under the following conditions: a SUPELCO ODS C_{18} column (150 cm × 4.6 mm; 5 μm particles, Bellefont, PA, USA) maintained at room temperature; an isocratic mobile phase which consisted of acetonitrile/methanol/phosphate buffer KH_2PO_4, 0.01 M (50 : 20 : 30, v/v) (pH 4.5); and a flow rate of 1 ml/min. The detective wavelength was set at 230 nm. The retention time was 4.9 min.

It was found that under these conditions, macrogol 6000, quinidine, naringin and tartaric acid did not interfere with the assay. Quantification of clopidogrel was carried out by measuring the peak areas in relation to those of standards chromatographs under the same conditions.

Statistical analysis

Statistical analysis was computed with Statgraphics Centurion XV. The results were expressed as mean ± SD of six independent experiments. A one-way analysis of variance test was used to compare the solubility, the apparent permeability (P_{app}) and the water flux average values of the different tested samples. The differences observed were considered significant at $P \leq 0.05$.

Results

Solubility studies

The aqueous solubility values of clopidogrel and clopidogrel-macrogol 6000 binary systems (physical mixtures and solid dispersions) at different drug:polymer weight ratios (1 : 1, 1 : 3, 1 : 5, 1 : 7 and 1 : 9, w/w) in pH 6.8 buffer solution are reported in Table 1.

Solubility value of clopidogrel was 16.56 ± 1.65 μg/ml. As shown in Table 1, there was a solubility enhancement with physical mixtures and solid dispersions at different drug:polymer weight ratios compared with pure drug ($P < 0.05$), except for the physical mixture at 1 : 1, 1 : 3 and 1 : 5 (w/w) ratios ($P > 0.05$). Compared with the physical mixtures, solid dispersions showed a significant solubility improvement for all drug:carrier ratios tested ($P < 0.05$). In both cases (physical mixtures and solid dispersions), solubility values increased with increasing macrogol 6000 weight fraction. For the physical mixtures, the lowest solubility

Table 1 Solubility values of pure clopidogrel and drug:carrier binary systems (physical mixtures and solid dispersions) at various weight ratios in pH 6.8 buffer solution

Sample	Clopidogrel-macrogol 6000 ratio (w/w)	Solubility (μg/ml)†
Clopidogrel	1 : 0	16.56 ± 1.65
Physical mixtures	1 : 1	17.49 ± 1.69
	1 : 3	17.55 ± 0.90
	1 : 5	18.41 ± 2.09
	1 : 7	*20.19 ± 3.09
	1 : 9	*22.08 ± 1.42
Solid dispersion	1 : 1	*142.21 ± 22.93
	1 : 3	*191.35 ± 26.60
	1 : 5	*256.15 ± 25.61
	1 : 7	*280.25 ± 7.12
	1 : 9	*354.88 ± 13.69

†Solubility data are presented as mean ± SD. *$P < 0.05$ compared with clopidogrel only.

value (17.49 ± 1.69 μg/ml) was obtained at 1 : 1 (w/w) clopidogrel-macrogol 6000 ratio, whereas the greatest value (22.08 ± 1.42 μg/ml) was recorded for 1 : 9 (w/w) clopidogrel-macrogol 6000 ratio. Similar conclusions were noticed for solid dispersions, where the lowest solubility value (142.21 ± 22.93 μg/ml) was noticed for 1 : 1 (w/w) drug:carrier weight ratio, while the highest value (354.88 ± 13.69 μg/ml) was observed for 1 : 9 (w/w) clopidogrel-macrogol 6000 ratio. For solid dispersions at a drug:polymer ratio 1 : 9 (w/w), we recorded a solubility enhancement of 21.44-fold compared with clopidogrel alone in pH 6.8 buffer medium.

Permeability studies

Apparent permeability coefficients P_{app} (cm/s), of clopidogrel (232 μM), clopidogrel-macrogol 6000 binary systems (physical mixture, solid dispersion) at drug:polymer weight ratio 1 : 9 (w/w), clopidogrel+quinidine (10, 50 or 200 μM), clopidogrel+naringin (5, 10 or 15 mg/kg), clopidogrel + tartaric acid (5, 10 or 20 mM), solid dispersions (1 : 9, w/w) + quinidine (200 μM) and (1 : 9, w/w) + naringin (15 mg/kg) across the everted gut sac are shown in Figure 1. Data are expressed as mean ± SD and results are expressed in cm/s.

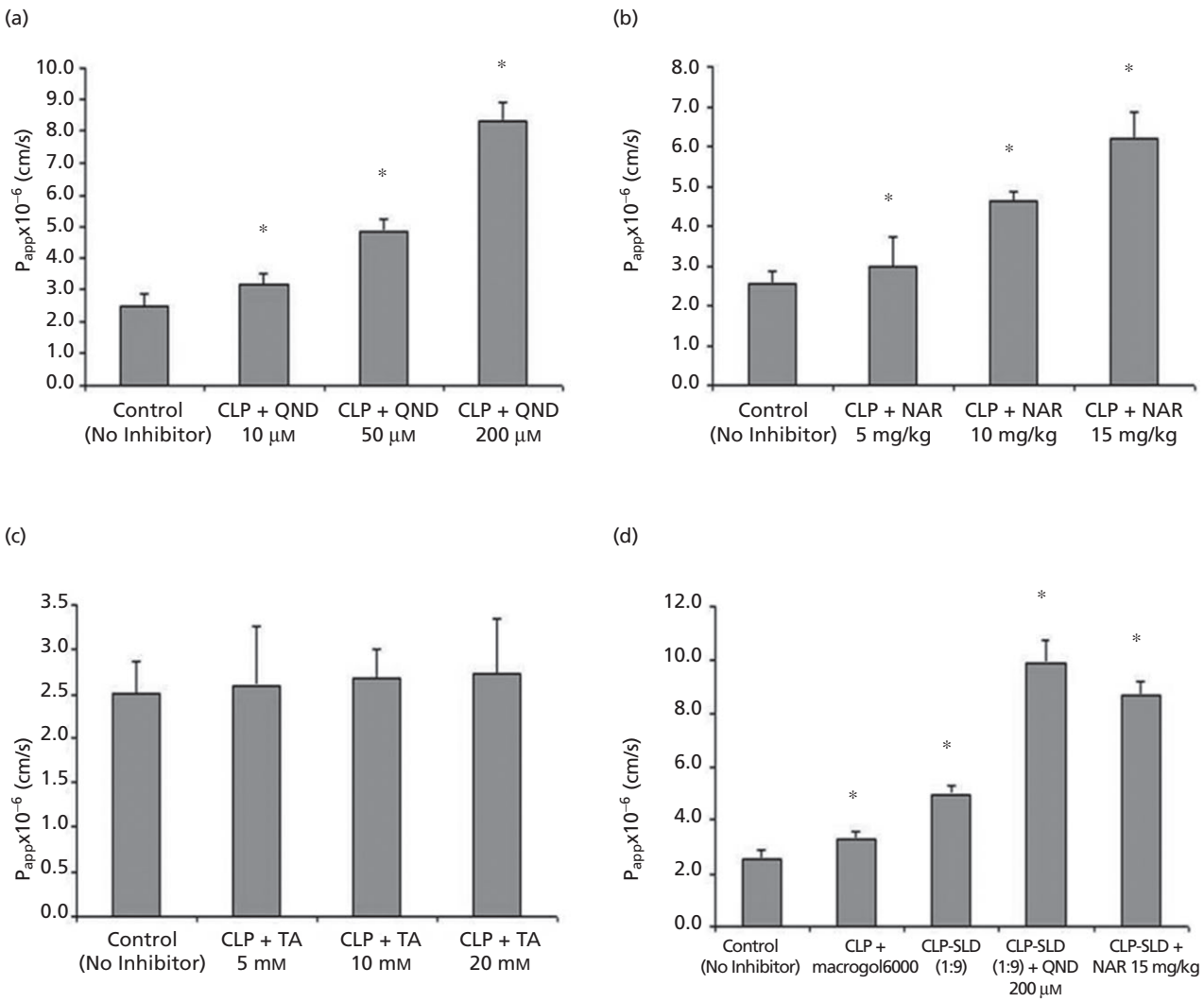

Figure 1 The apparent permeability values (P_{app}) of clopidogrel across everted gut sac model. Clopidogrel (CLP; 232 μM) in the presence of (a) quinidine (QND; 10, 50 or 200 μM), (b) naringin (NAR; 5, 10 or 15 mg/kg), (c) tartaric acid (TA; 5, 10 or 20 mM), and (d) clopidogrel-macrogol 6000 binary systems (CLP-macrogol 6000) at drug-polymer weight ratio 1 : 9 (w/w) in absence or presence of naringin (15 mg/kg) or quinidine (200 μM). SLD, solid dispersion. Data are presented as mean ± SD; $n = 6$ in each experimental group. *$P < 0.05$ compared with control.

The apparent permeability value (P_{app}) of clopidogrel was $2.51 \pm 0.34 \times 10^{-6}$ cm/s. In the presence of P-gp inhibitors at various concentrations (10, 50 and 200 μM quinidine or 5, 10 and 15 mg/kg naringin) there was a significant increase of clopidogrel P_{app} values ($P \leq 0.05$). The greatest enhancements were achieved in presence of 200 μM quinidine ($8.33 \pm 0.58 \times 10^{-6}$ cm/s) and 15 mg/kg naringin ($6.18 \pm 0.68 \times 10^{-6}$ cm/s). However, no significant increase in clopidogrel apparent permeability was noticed in presence of tartaric acid at any of the concentrations tested (5, 10 and 20 mM) ($P \geq 0.05$). P_{app} coefficients recorded were $2.59 \pm 0.66 \times 10^{-6}$, $2.67 \pm 0.34 \times 10^{-6}$ and $2.72 \pm 0.62 \times 10^{-6}$ cm/s with 5, 10 and 20 mM tartaric acid, respectively.

For clopidogrel-macrogol 6000 binary systems (physical mixtures and solid dispersions) a significant increase in apparent permeability coefficients ($P \leq 0.05$) was noted. The lowest P_{app} value was observed with the physical mixture of clopidogrel and macrogol 6000 ($3.33 \pm 0.25 \times 10^{-6}$ cm/s), whereas the highest P_{app} values ($9.85 \pm 0.88 \times 10^{-6}$ and $8.68 \pm 0.49 \times 10^{-6}$ cm/s) were found with solid dispersions in presence of 200 μM quinidine or 15 mg/kg naringin, respectively. The P_{app} coefficient of a solid dispersion (1 : 9; w/w) in absence of P-gp inhibitors (quinidine or naringin) was $4.95 \pm 0.34 \times 10^{-6}$ cm/s.

Percentage of drug recovery and drug retained determination

The percentages of drug recovery (*R*%) and drug retention (*Ad*%) of clopidogrel (232 μM), clopidogrel-macrogol 6000 binary systems (physical mixture, solid dispersion) at drug : polymer weight ratio 1 : 9 (w/w), clopidogrel in presence of quinidine (10, 50 or 200 μM), naringin (5, 10 or 15 mg/kg) or tartaric acid (5, 10 or 20 mM) and solid dispersions (1 : 9 w/w) in presence of quinidine (200 μM) or naringin (15 mg/kg) across the everted gut sac model are shown in Table 2. Results are expressed as mean ± SD ($n = 6$). For all experiments, the percentages of drug retention on the intestinal barrier were below 10%.

Diffusion profiles comparison

Figure 2 shows the absorption rates of clopidogrel (232 μM) across the everted gut sac model in the presence of quinidine at various concentrations (10, 50 or 200 μM; Figure 2a); naringin at different administered doses (5, 10 or 15 mg/kg; Figure 2b); tartaric acid (5, 10 or 20 mM; Figure 2c); and the absorption rates of clopidogrel in the clopidogrel-macrogol 6000 binary systems at drug/carrier ratio 1 : 9 (w/w) in the absence or presence of P-gp inhibitors (200 μM quinidine or 15 mg/kg naringin; Figure 2d). Data are expressed as mean ± SD ($n = 6$) and results are expressed as a percentage.

Diffusion studies showed that a significant increase in the transport of clopidogrel (232 μM) from outside to inside the everted gut sacs occurred in the presence of quinidine (10, 50 and 200 μM), naringin (5, 10 or 15 mg/kg) and for clopidogrel-macrogol 6000 binary systems (physical mixtures and solid dispersions 1 : 9, w/w)) ($P \leq 0.05$), whereas no significant enhancements were observed when tartaric acid (5, 10 or 20 mM) was added to the medium ($P \geq 0.05$).

Indeed, after the experiment had run for 120 min, we noticed an improvement of 1.41-, 1.95- and 3.44-fold for clopidogrel absorption in the presence of 10, 50 and 200 μM quinidine, respectively. For naringin 5, 10 or 15 mg/kg, an improvement of 1.16-, 1.94- and 2.38-fold was observed, respectively. Finally, for clopidogrel-macrogol 6000 binary systems (1 : 9; w/w), an increase of 1.46-, 1.96-, 3.56- and 3.85-fold in clopidogrel absorption was noticed for the physi-

Table 2 Percentages of drug recovery and drug retention for clopidogrel (232 μM) across the everted gut sac model in the presence of quinidine, naringin, tartaric acid, and clopidogrel-macrogol 6000 binary systems at drug-polymer weight ratio 1 : 9 (w/w) in absence or presence of naringin or quinidine

In vitro absorption model	Sample	Percentage of drug recovery (%)*	Percentage of drug retention (%)*
Everted gut sac technique	Clopidogrel (control)	92.65 ± 1.74	7.35 ± 1.74
	Clopidogrel + quinidine (10 μM)	92.73 ± 1.21	7.27 ± 1.21
	Clopidogrel + quinidine (50 μM)	92.80 ± 1.31	7.20 ± 1.31
	Clopidogrel + quinidine (200 μM)	93.09 ± 1.64	6.91 ± 1.64
	Clopidogrel + naringin (5 mg/kg)	92.18 ± 1.04	7.82 ± 1.04
	Clopidogrel + naringin (10 mg/kg)	93.24 ± 1.38	6.76 ± 1.38
	Clopidogrel + naringin (15 mg/kg)	92.95 ± 1.83	7.05 ± 1.83
	Clopidogrel + tartaric acid (5 mM)	93.78 ± 1.73	6.22 ± 1.73
	Clopidogrel + tartaric acid (10 mM)	94.44 ± 1.74	5.56 ± 1.74
	Clopidogrel + tartaric acid (20 mM)	93.80 ± 1.17	6.20 ± 1.17
	Clopidogrel+macrogol 6000 (1 : 9, w/w)	93.73 ± 1.37	6.27 ± 1.37
	Clopidogrel-solid dispersion (1 : 9, w/w)	92.81 ± 1.56	7.19 ± 1.56
	Clopidogrel-solid dispersion (1 : 9, w/w) + quinidine (200 μM)	92.20 ± 1.39	7.80 ± 1.39
	Clopidogrel-solid dispersion (1 : 9, w/w) + naringin (15 mg/kg)	93.10 ± 1.44	6.90 ± 1.44

*Data are presented as mean ± SD; ($n = 6$).

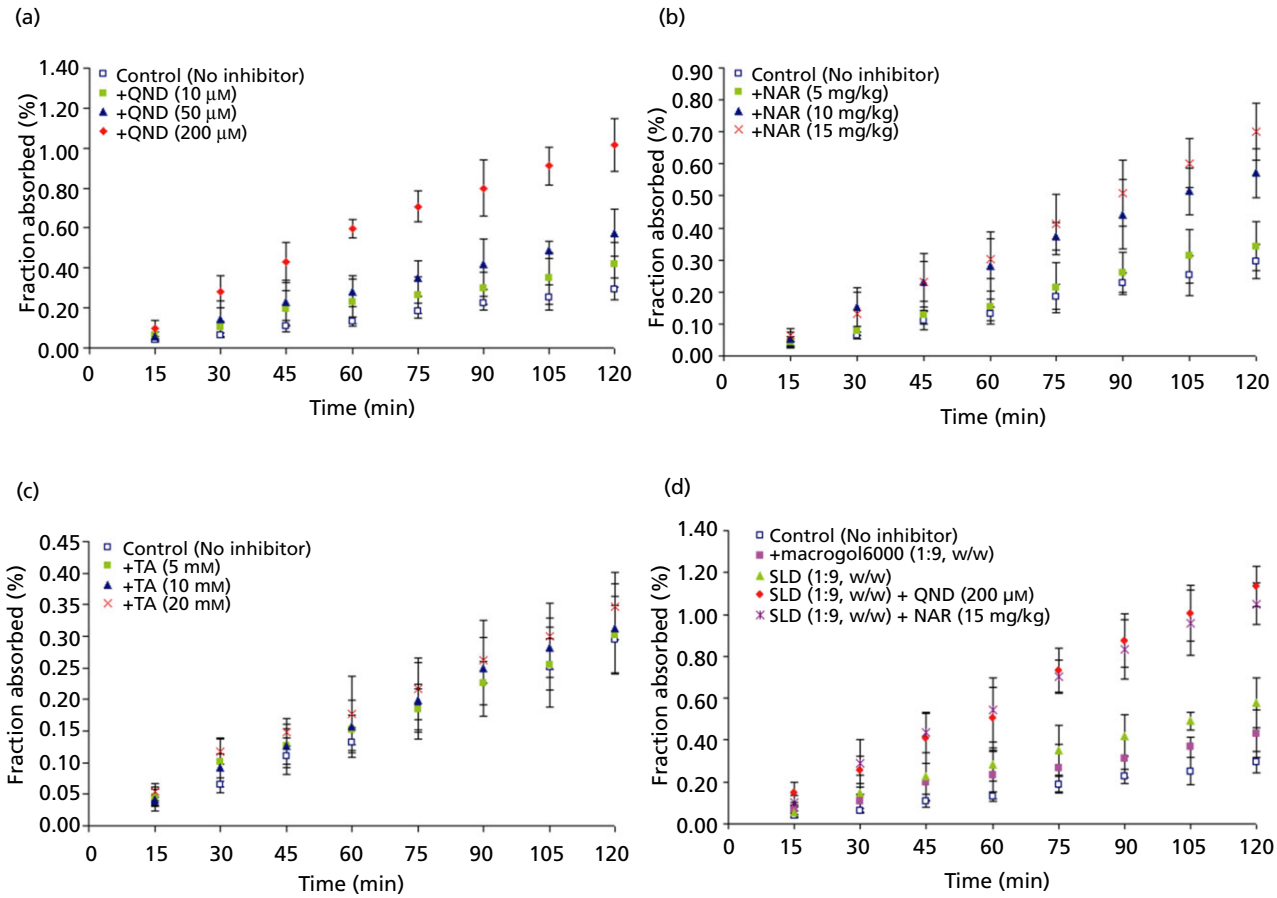

Figure 2 Absorption rates of clopidogrel across the everted gut sac model. Clopidogrel in presence of (a) quinidine (QND; 10, 50 or 200 μM), (b) naringin (NAR: 5, 10 or 15 mg/kg), (c) tartaric acid (TA; 5, 10 or 20 mM), and (d) clopidogrel-macrogol 6000 binary systems at drug-polymer weight ratio 1 : 9 (w/w) in absence or presence of naringin (15 mg/kg) or quinidine (200 μM). SLD, solid dispersion. Data are presented as mean ± SD; $n = 6$ in each experimental group.

cal mixture, solid dispersions, solid dispersion + quinidine (200 μM) and solid dispersion + naringin (15 mg/kg), respectively. However, in the presence of tartaric acid we noted a slight but not significant improvement in clopidogrel passage across the intestinal barrier compared with clopidogrel alone (1.04-, 1.06- and 1.18-fold enhancement in the presence of 5, 10 or 20 μM tartaric acid, respectively).

The highest fraction absorbed values (%) after 2 h of the experiment were obtained for clopidogrel in the presence of 200 μM quinidine (1.01 ± 0.13%) or 15 mg/kg naringin (0.70 ± 0.09%) and for clopidogrel-solid dispersion (1 : 9; w/w) in presence of 200 μM quinidine (1.14 ± 0.10%) or 15 mg/kg naringin (1.05 ± 0.10%).

Table 3 shows the results of the difference factor (f_1) and the similarity factor (f_2) for the diffusion profiles obtained with: clopidogrel vs clopidogrel+tartaric acid at various doses (5, 10 or 20 mM); and solid dispersions+quinidine (200 μM) vs solid dispersion+naringin (15 mg/kg). The f_1 and f_2 values were less than 15% and higher than 50%, respectively, when comparing the diffusion curves of clopidogrel vs clopidogrel + tartaric acid at 5, 10 or 20 mM. Similar finding were noticed in the case of solid dispersion + quinidine (200 μM) compared with solid dispersion+naringin (15 mg/kg), where f_2 and f_1 were 88.06 % ($\geq$ 50%) and 3.28 % ($\leq$ 15%), respectively.

Water flux studies

Figure 3 represents the variation of water flux after 2 h of experiments, expressed in g water/g fresh intestine, for clopidogrel in the absence (control) or in the presence of quinidine, naringin or tartaric acid used at various concentrations, for physical mixtures (1 : 9; w/w) and for solid dispersions (1 : 9; w/w) in the absence or presence of quinidine (200 μM) or naringin (15 mg/kg). During all the experiments we noticed a transfer of water from outside (mucosal side) to

Table 3 The difference factor and the similarity factor values for the diffusion profiles of clopidogrel and clopidogrel formulated with P-glycoprotein inhibitor or absorption enahncer obtained with the everted gut sac model

Drug	Difference factor (f_1) (%)	Similarity factor (f_2) (%)
Clopidogrel vs clopidogrel + tartaric acid (5 mM)	2.20%	93.03%
Clopidogrel vs clopidogrel + tartaric acid (10 mM)	8.40%	92.47%
Clopidogrel vs clopidogrel + tartaric acid (20 mM)	12.46%	91.94%
Clopidogrel-macrogol 6000 solid dispersion at drug:polymer weight ratio 1 : 9 (clopidogrel-solid dispersion) + quinidine (200 μM) versus clopidogrel-solid dispersion+naringin (15 mg/kg)	3.28%	88.06%

inside (serosal side) the everted gut sacs, which we translated as showing absorption. The addition of quinidine (10, 50 or 200 μM) or naringin (5, 10 or 15 mg/kg) in the mucosal medium provoked a significant increase in water influx ($P < 0.05$). Similar observations were found for clopidogrel-macrogol 6000 systems in comparison with the control ($P < 0.05$). However, no significant water flux differences were noticed between clopidogrel + tartaric acid at the various concentrations (5, 10 and 20 mM) and clopidogrel alone ($P \geq 0.05$).

After 120-min incubation, water flux was 0.74 ± 0.19 g water/g fresh intestine for clopidogrel alone. In the presence of 10, 50 or 200 μM quinidine, water flux values were 0.92 ± 0.13, 1.12 ± 0.14 and 1.38 ± 0.36 g water/g fresh intestine, respectively. After the addition of 5, 10 or 15 mg/kg naringin in the mucosal medium, water flux values noted were 0.86 ± 0.12, 1.13 ± 0.13 and 1.25 ± 0.32 g water/g fresh intestine, respectively. With 5, 10 or 20 mM tartaric acid added to the outside of the everted gut sac, water flux values were 0.78 ± 0.12, 0.79 ± 0.13 and 0.81 ± 0.31 g water/g fresh intestine, respectively. Finally, with clopidogrel-macrogol 6000 binary systems, water flux results were 0.90 ± 0.23, 1.01 ± 0.26, 1.53 ± 0.29 and 1.45 ± 0.22 g water/g fresh intestine for physical mixture, solid dispersion, solid dispersion + quinidine (200 μM) and solid dispersion + naringin (15 mg/kg), respectively.

Discussion

In this study, we attempted to improve the intestinal absorption of clopidogrel by enhancing its solubility and by modulating the efflux pump P-gp activity.

Clopidogrel solubility improvement was achieved by preparing solids dispersions using macrogol 6000 as a hydrophilic carrier. There are two main processes for preparing solid dispersions, the melting and the solvent evaporation methods.[12] In this study, clopidogrel solid dispersions were obtained using the solvent evaporation method, since this method prevents the thermal decomposition of both drug compound and hydrophilic polymer compared with the melting method (solvent evaporation occurs at low temperatures).[12] Macrogol 6000 was chosen as carrier because of its low toxicity and its wide drug compatibility.[24] Finally, we used a mixture of water-ethanol as solvent because it is less toxic then other solvents which may be used to prepare solid dispersions by the solvent evaporation method such as dichloromethane.

Clopidogrel solubility value was 16.56 ± 1.65 μg/ml in pH 6.8 buffer solution medium. This result was in good accordance with published data.[21,25] For the physical mixtures and solid dispersions it could be noticed that clopidogrel solubility increase was related to clopidogrel and macrogol 6000 weight ratios. The greatest improvements in terms of clopidogrel solubility were achieved at the clopidogrel:macrogol 6000 weight ratio 1 : 9 in both cases. At this ratio, we recorded an enhancement of 1.33- and 21.44-fold for the physical mixture and solid dispersion, respectively, in comparison with clopidogrel alone. For the physical mixture (1 : 9; w/w), the improvement was not significant ($P > 0.05$), while for the solid dispersion, a considerable increase in clopidogrel solubility occurred ($P < 0.05$). This solubility enhancement may have been attributed to several factors, namely drug particle size reduction, the drug wettability and dispersibility enhancement and the absence of crystal structure in solid dispersions; in addition to the great water solubility of macrogol 6000, it also has a solubilizing effect and tension surface lowering effect between the hydrophobic drug and the buffer solution medium.[24,26]

Clopidogrel absorption assessment was performed with the in-vitro everted gut sac model prepared from rat jejunum. Over the years, numerous in-situ, in-vivo, ex-vivo and in-vitro models have been developed to predict drug permeability and to assess the impact of influx and efflux transporters such as P-glycoprotein (P-gp) on drug absorption.[27,28] The everted gut sac model is advantageous since it is a simple, rapid and economic method. Moreover, it has been shown, in previous studies, that this technique can be used as an efficient tool to assess P-gp role in the intestinal absorption of a wide range of drug compounds.[29–31] The validity of this in-vitro model by histological studies was explored in published data.[22,32,33] Histological slides of

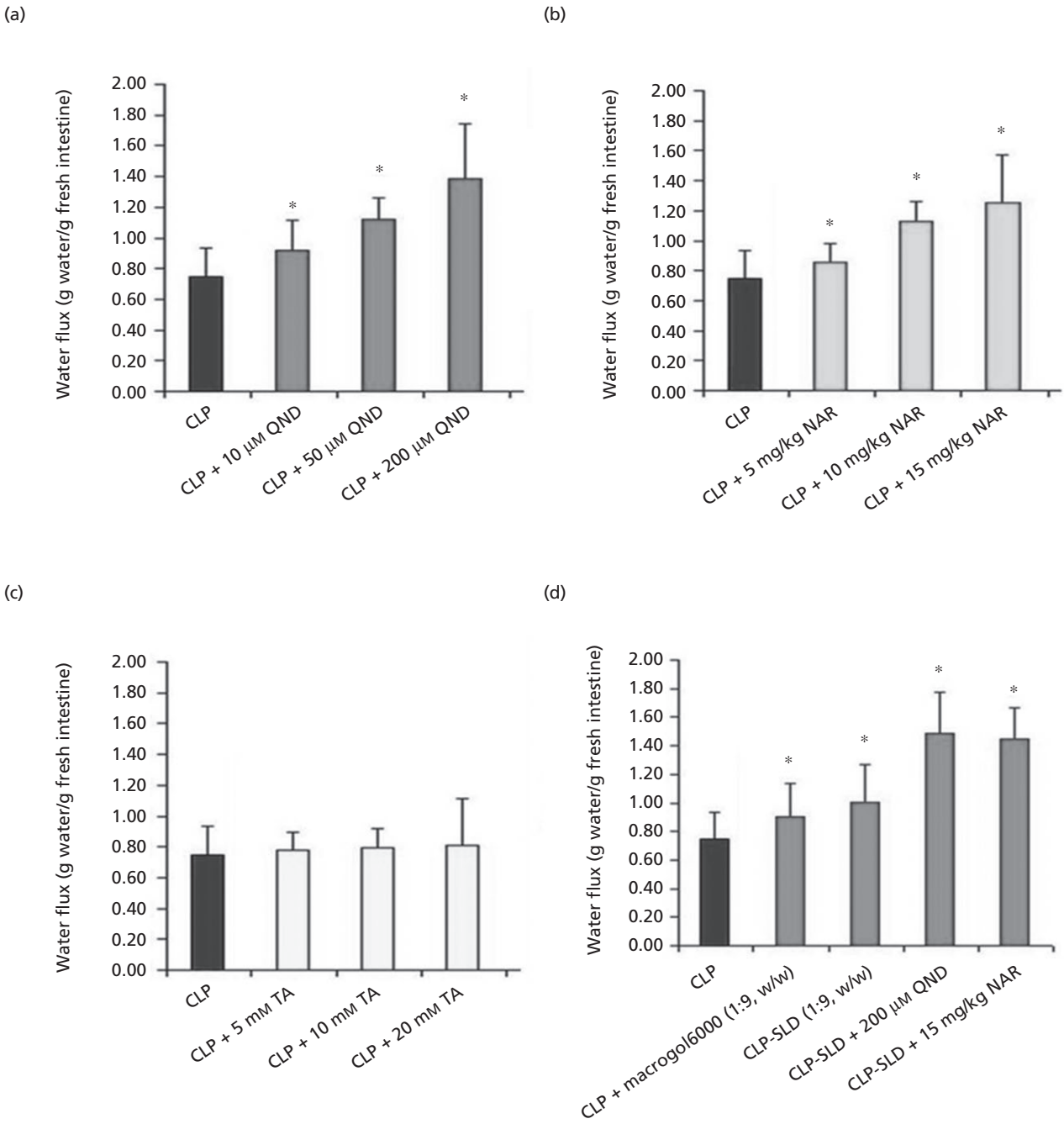

Figure 3 Variation of water flux in the everted gut sacs of rat jejunum. (a) Clopidogrel (CLP) + quinidine (QND: 10, 50 or 200 μM), (b) clopidogrel + naringin (NAR: 5, 10 or 15 mg/kg), (c) clopidogrel + tartaric acid (TA; 5, 10 or 20 mM), and (d) clopidogrel-macrogol 6000 binary systems at drug-polymer weight ratio 1 : 9 (w/w) in absence or presence of quinidine (200 μM) or naringin (15 mg/kg) after 2 h of experiments. SLD, solid dispersion. Control group was everted gut sacs containing pure clopidogrel. $n = 6$. $^*P < 0.05$ compared with control group.

intestine observed by light microscopy after 2 h of experiment showed that the intestine remained intact morphologically with a high number of villosities, microvillosities and intact brush border. Jejunum excision was made in anaesthetized rats before they were killed by cervical dislocation to avoid the deterioration of tissues and active transport.[34]

Permeability studies were undertaken for both clopidogrel and clopidogrel-macrogol 6000 binary systems at the drug : polymer weight ratio 1 : 9 (w/w; physical mixture and solid

dispersions) in the absence or presence of P-gp inhibitors, namely quinidine, an antiarrythmic agent, and naringin, a flavonoid which is the main constituent in grapefruit juice. We investigated the effect of tartaric acid, an absorption enhancer, on clopidogrel absorption. Quinidine, naringin and tartaric acid were tested at various concentrations taken from literature.[6,35,36]

For physical mixtures, clopidogrel permeability was enhanced 1.30-fold compared with clopidogrel alone. This enhancement may have been attributed to the slight solubility enhancement of clopidogrel in the presence of macrogol 6000 and also to the role that macrogol 6000 can play as a P-gp modulator. In fact, previous studies demonstrated that macrogols have a modulating effect, concentration-dependant, on intestinal absorption of P-gp substrates.[17,37] In the case of solid dispersions, we noted a significant enhancement of clopidogrel permeability compared with clopidogrel alone (1.97-fold). This great increase may have been attributed to the solubility improvement of clopidogrel in solid dispersions and to the modulating effect of macrogol 6000 on the P-gp efflux pump.[17,38]

The addition, in the mucosal medium, of quinidine or naringin at various concentrations in the presence of clopidogrel alone or solid dispersion (1 : 9; w/w) increased markedly the permeability values of the drug ($P < 0.05$). This could be attributed to the inhibitory effect of those compounds on the P-gp efflux transporter and also to the inhibitory effect of naringin on the intestinal cytochrome P450 (CYP) 3A4 isoenzyme activity.[6,11,17,35] Data from our study concerning the effect of quinidine on P-gp activity were consistent with a study by Taubert *et al*.[6] on the assessment of the impact of P-gp mediated efflux on clopidogrel absorption. In that study performed using the in-vitro Caco-2 monolayers model, they found that clopidogrel permeability was increased by approximately 5-fold in the presence of 200 μM quinidine compared with clopidogrel alone. In our case, we noticed that in the presence of quinidine at the same concentration, the apparent permeability coefficient was increased by approximately 3-fold. The difference in results observed between the Caco-2 and the everted gut sac models may have been attributed to the overexpression of P-gp on the Caco-2 cells resulting in a greater improvement on clopidogrel absorption in the presence of quinidine.[29] As can be noticed from the clopidogrel absorption data obtained when quinidine or naringin were added to the mucosal medium, the P-gp activity inhibition was concentration-dependant in both cases. When these two P-gp inhibitors were used at their highest concentrations (200 μM quinidine and 15 mg/kg naringin), we recorded an increase in clopidogrel apparent permeability values by 3.30- and 3.91-fold in the presence of quinidine, and by 2.46- and 3.46-fold in the presence of naringin for clopidogrel alone and for the solid dispersion (1 : 9; w/w), respectively. As can be seen from these results, enhancing clopidogrel solubility and inhibiting P-gp activity at the same time led to the best results in terms of clopidogrel permeability improvement. Clopidogrel diffusion studies undertaken in the presence of quinidine (200 μM) or naringin (15 mg/kg) were compared using a model-independent approach consisting of the calculation of the difference factor (f_1) and the similarity factor (f_2). f_1 values less than 15% and f_2 values greater than 50% ensure equivalence of the two curves.[23] Thus we can consider that the diffusion profiles obtained in the presence of these two P-gp inhibitors were similar ($f_1 = 3.28\%$ (< 15%) and $f_2 = 88.06\%$ (> 50%); Table 3).

These results were interesting since quinidine has pharmacological activity and its use as a P-gp inhibitor may lead to toxic side effects, whereas, naringin is a natural component which can be used without a risk to health.

In the case of tartaric acid, the results of permeability studies obtained for clopidogrel in the presence of this absorption enhancer at various concentrations (5, 10 and 20 mM) showed that no significant increase occurred compared with control (clopidogrel alone) ($P \geq 0.05$). The comparison of diffusion profiles obtained for clopidogrel versus clopidogrel+tartaric acid at all concentrations used showed that diffusion curves were similar ($f_1 < 15\%$ and $f_2 > 50\%$ in all three cases), which translated to clopidogrel absorption was not enhanced in the presence of tartaric acid. Tartaric acid is known to be a paracellular enhancer acting by enlargement of the tight junction. It increases the paracellular permeability of hydrophilic and low hydrophobic compounds. Tartaric acid activity on tight junction integrity is observed at low pH values but not at neutral pH. Opening tight junctions is generally observed in the colon but not in jejunum or ileum.[36,39] It has been reported that tartaric acid enhanced rhodamine 123 and daunorubicin absorption, two P-gp substrates.[36] This enhancement occurred in colon and ileum, but not in jejunum. Those authors suggested that the effects of tartaric acid on rhodamine 123 and daunorubicin absorption may not have been attributable to P-gp inhibition, but to the activation of absorption transporters or the inhibition of an efflux mechanism other than P-gp by tartaric acid. They suggested that the difference in results observed for the different regions of intestine (jejunum, ileum and colon) may have been due to the different distribution of dicarboxylate transporter in the intestine. Those hypotheses could explain the non-effect of tartaric acid on clopidogrel absorption enhancement.

In this study, we determined the water flux for clopidogrel and clopidogrel-macrogol 6000 binary systems in the presence of P-gp inhibitors (quinidine and naringin) and absorption enhancer (tartaric acid) at various drug concentrations. We found that, in all cases, there was a transfer of water from the outside to the inside of the sacs, which translated as absorption. The comparison of the water flux values for clopidogrel vs clopidogrel in the presence of quinidine,

tartaric acid or naringin, or clopidogrel vs clopidogrel-macrogol 6000 binary systems at the end of the assays (after 120 min) showed that the water flux was greater for clopidogrel in the presence of P-gp inhibitors or clopidogrel-macrogol 6000 binary system ($P < 0.05$) than for clopidogrel in the presence of tartaric acid ($P \geq 0.05$) in comparison with control (clopidogrel alone). This could be explained by the fact that the absorption of clopidogrel was affected by the intestinal efflux transporter P-gp. In fact, in previous studies concerning the role of P-gp in intestinal absorption of digoxin, Sababi *et al.*[40] reported that the water absorption increased significantly in the duodenum and the jejunum with verapamil, an inhibitor of P-gp.

During permeation experiments undertaken with the everted gut sac model, it was important to assess the percentages of clopidogrel recovery (*R*%) outside and inside the sacs and the percentage of clopidogrel retained (*Ad*%) on the intestinal tissue. The results showed that, for all experiments, the values for *R*% were above 90%, indicating that the amount of drug retained by the everted gut sac tissues was very limited. This phenomenon was reported previously, and may have been due to the fact that during the passage from mucosal to serosal side on the everted gut sac, clopidogrel must cross the whole intestinal wall, which can lead to accumulation of the drug into the muscular layer.[20]

Conclusions

Clopidogrel is a poor water soluble compound and P-gp efflux pump substrate, which explains its incomplete intestinal absorption after oral administration. In this study, we have tried to overcome these problems by enhancing clopidogrel solubility via solid dispersions prepared with macrogol 6000, and by inhibiting P-gp activity using quinidine or naringin as inhibitors, and tartaric acid as an absorption enhancer. Results showed that clopidogrel intestinal absorption could be increased, possibly by enhancing clopidogrel solubility, or by modulating the P-gp efflux pump activity via P-gp inhibitors, namely quinidine and naringin. The best results in terms of clopidogrel intestinal diffusion were obtained by combining solubility enhancing and P-gp inhibition. Results showed that P-gp inhibition was comparable in the presence of quinidine (200 μM) or naringin (15 mg/kg). These findings were interesting because it is well known that the main P-gp inhibitors reported in literature are pharmacologically active substances (such as quinidine or verapamil), and their use leads to undesirable side effects, which may be dangerous. Thus, the use of natural components that do not present a health risk, such as naringin, as inhibitors of P-gp, in association with clopidogrel-macrogol 6000 solid dispersions to increase the absorption of clopidogrel in the intestine, and hence its oral bioavailability, are of considerable interest. These in-vitro results are promising and in-vivo studies are necessary to support this study.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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